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PATHOGENESIS AND PREVENTION OF ACUTE RENAL FAILURE

Final Report

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Calcium channel blockers (CCB) including verapamil, nifedipine and emopamil, and calmodulin antagonists W-7 and trifluoperazine administered 1) in vivo, 2) in the isolated perfused kidney, 3) to isolated proximal tubules, or 4) to cultured tubules prevent or greatly attenuate O_2 deprivation injury. Although the absolute decrease in adenosine triphosphate (ATP) and total adenine nucleotides that occurs in O_2 deprivation injury is well known, this does not appear to be the sole or primary cause of injury since similar decreases can be achieved with glycerol or fructose and no injury is seen. However, the preservation of cellular ATP seen with fructose diphosphate treatment and the rapid return of ATP and adenine nucleotide levels to near normal is important in enhancing the recovery of renal function. Finally, verapamil and possibly other CCB may exert their effect on hypoxic tissue by reducing Ca uptake. The cellular accumulation of Ca during reoxygenation still is the most prevalent mediator of injury since blockade of this event with CCB, calmodulin binding drugs or low media Ca can all attenuate cell death. AP-III also exerts powerful protective effects on GFR in the intact rat previously exposed to a period of

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19. Abstract (continued)

ischemia. The isolated perfused rat kidney was used to evaluate the protective effect of AP-III and, as in the intact rat, the principal effect was on improving GFR. Based on these impressive experimental results, we have begun studies in patients to evaluate the efficacy of using AP-III and calcium entry blockers to prevent ischemic (and other forms of) acute renal failure.

SUMMARY

Hypoxic and anoxic injury in isolated renal tubules, cultured renal tubule cells and in vivo were studied intensely during this study. Clearly, hypoxic injury is associated with increased $^{45}Ca^{2+}$ uptake and this can be prevented extracellular acidosis (pH 6.9). O_2 deprivation injury can be prevented by calcium channel blockers, calmodulin antagonists, administration of fructose diphosphate, and atriopeptin III (AP-III) Preliminary studies using phospholipase inhibitors and allopurinol suggest additional maneuvers that also lessen the severity of renal injury. Together these agents may exert protection which is synergistic or additive since alone each modulates injury to a variable degree. Finally, we have also begun studies in patients with acute renal failure using the experimental data obtained during this contract as a basis for the intrarenal administration of gallopamil (D600) and AP-III (atrial natriuretic factor).

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

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The purpose of this contract was to investigate the pathogenesis of ischemic acute renal failure (ARF) and to evaluate maneuvers which would prevent this abrupt deterioration of renal function. Our investigations were directed primarily at the role of Ca ions in mediating the functional, biochemical and morphologic injury that characterizes ARF. We have used intact rats, the isolated perfused kidney preparation, freshly isolated rat renal proximal tubules, and cell culture of precisely defined segments of rabbit proximal and distal tubules. Based upon the results of these studies, we have written a protocol to be investigated in humans and initiated studies in patients at risk for ARF. The report to follow will highlight the experiments conducted during this contract.

I. Prevention of Cellular Injury

Before the initiation of this contract we had shown that verapamil prevents cold and warm ischemic renal injury as assessed by inulin clearance, and tubular sodium reabsorption (1). During the present contract, sequential warm and cold ischemia were induced in the same model and this model was used to examine the protective utility of emopamil, a verapamil analogue with little cardiodepressant effect. The data show that emopamil has a salutory effect on kidney function following warm and cold ischemia (2). The following table (from Ref. 2) identifies the protective effect of emopamil which normalized renal plasma flow, tripled inulin clearance and increased tubular reabsorption compared to ischemia alone. The low effective dose of emopamil, as demonstrated in this study make this compound an exciting new calcium channel blocker (CCB) to be used in the prevention of ischemic injury.

Continuous cold perfusion has been used as a method of preservation in clinical practice in addition to simple ice storage. Therefore, we have extended this study to include the use of verapamil in long-term continuous cold perfusion in the isolated rat kidney as the method of organ preservation. Renal functional performance was improved by the addition of verapamil to the cold perfusate (3). The following tables (from reference 3) describe 1) the improvement in renal function including inulin clearance, fractional sodium reabsorption and tissue ATP content during reperfusion, and 2) the beneficial effects on glomerular filtration when verapamil is added to the cold perfusion media.

TABLE 1. Effects of Emopamil on renal function following warm and cold ischemia[a]

Group	(N)	RPF (ml/min/g)	C_{in} (μl/min/g)	UV (μl/min/g)	FR_{NA}* (%)
Normal (no ischemia)	(7)	39±2	492±63	14±3	99±1
Control	(11)	33±1	58±7	30±4	48±4
Emopamil	(7)	39±1[b]	146±23[b]	34±5	76±4[b]

* Results, expressed as mean ± SEM for each kidney, are the average values of three 15-min collection periods during the last 45 min of reperfusion following a 15-min warm ischemic renal artery clamp and 4 hr of cold ischemia.

[b] $P<.01$ compared with control. Normal values (kidneys perfused without any warm or cold ischemia) are shown for comparison.

TABLE 1. Renal function during warm reperfusion[a]

	CP4 (n=6)[b]	CP24 (n=9)[c]	CP24V (n=5)[d]	SS4 (n=6)[e]	SS24 (n=4)[f]
RPF (ml/min/g)	34 ± 1	32 ± 2	33 ± 1	30 ± 2	29 ± 1
C_{in} (μl/min/g)	511 ± 42	168 ± 20	271 ± 30[g]	121 ± 39	0
$\dot{V}$ (μl/min/g)	49 ± 9	71 ± 11	55 ± 13	23 ± 5	0
$T_{Na}+$ (μmol/min/g)	65 ± 5	15 ± 3	32 ± 6[g]	13 ± 5	0
$FR_{Na}+$ (%)	96 ± 1	57 ± 8	84 ± 5[g]	70 ± 11	0
ATP (μmol/g dry wt.)	7.1 ± 0.4	4.7 ± 1.0	8.0 ± 0.5[g]	6.0 ± 0.6	1.7 ± 0.3

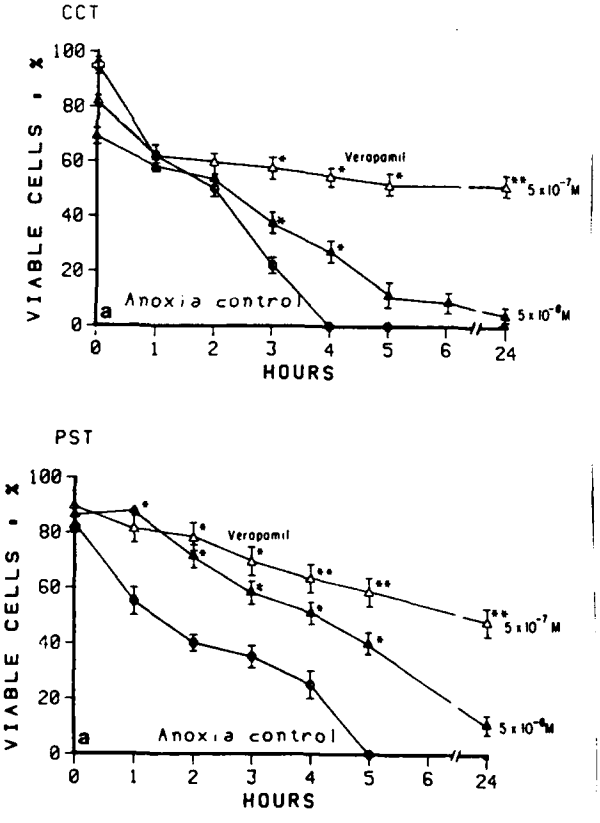
[a] Results expressed as mean ± SEM.
[b] CP4: 4-hr cold perfusion.
[c] CP24: 24-hr cold perfusion.
[d] CP24V: 24-hr cold perfusion with verapamil.
[e] SS4: 4-hr simple storage.
[f] SS24: 24-hr simple storage.
[g] $P<0.01$ vs. CP24.

TABLE 2. Renal function during 24-hr cold perfusion[a]

	24 hr cold perfusion		24 hr cold perfusion with verapamil	
	First 4 hr (n=9)	Last 4 hr (n=9)	First 4 hr (n=5)	Last 4 hr (n=5)
RPF (ml/min/g)	9 ± 1	17 ± 1	10 ± 1	16 ± 2
C_{in} (μl/min/g)	34 ± 7	35 ± 8	114 ± 18	88 ± 14
$\dot{V}$ (μl/min/g)	15 ± 9	20 ± 5	28 ± 10	54 ± 11
$T_{Na}+$ (μmol/min/g)	4 ± 1	2 ± 1[b]	13 ± 2	6 ± 1[c]
$FR_{Na}+$ (%)	80 ± 3	51 ± 5[c]	81 ± 5	48 ± 4[c]

[a] Results expressed as mean ± SEM.
[b] $P<0.05$ vs. first 4 hr.
[c] $P<0.01$ vs. first 4 hr.

These investigations into the role of CCB in isolated perfused kidneys subjected to warm and cold ischemia indicated that tubular function (fractional sodium reabsorption) was improved and could potentially be a direct effect of the CCB. To investigate this latter possibility, we dissected individual nephron segments of the rabbit nephron and cultured these for 7 days in hormonally defined media (4). The data for proximal tubules (shown below) and that for distal tubules, cortical collecting tubules are nearly identical. Both verapamil and nifedipine (data not shown; see Ref. 4) delayed the onset of anoxic cell death and furthermore both W-7 and trifluoperazine (TFP), putative calmodulin antagonists were also protective (see following figures from reference 4).



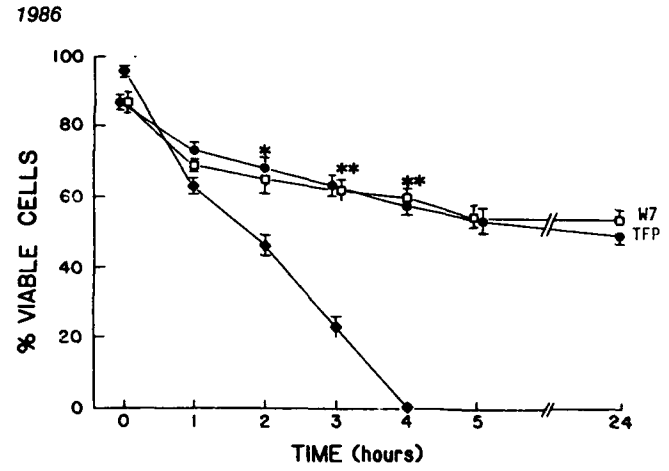


Fig. 5. Effect of anoxia on cultured CCT cells. Conditions and drugs are the same as in figure 4.

In summary, in three different models of O_2 deprivation injury we described the protective effect of CCB including verapamil, emopamil and nifedipine and the similar protective effect of calmodulin antagonists. The isolated cultured tubule studies clearly implicate a direct protective role for CCB on tubular viability after an anoxic or ischemic insult. The suggested

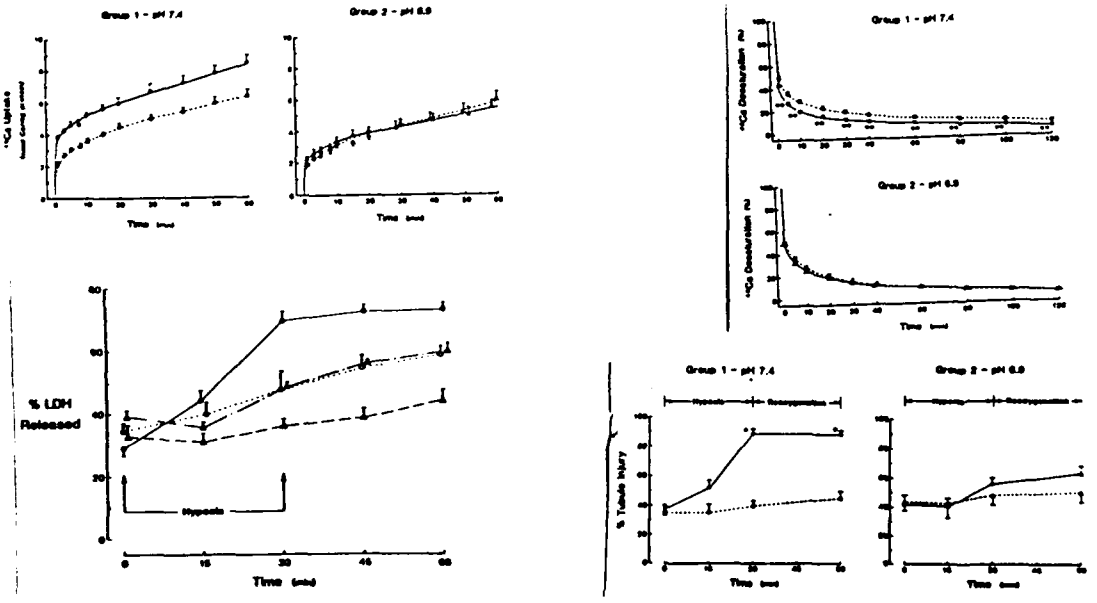
role of Ca ions in the pathogenesis of injury is further supported by the enhanced viability seen when Ca-calmodulin antagonism is achieved.

Our efforts in this area which strongly implicate a causal role for Ca ions in mediating ischemic injury, led us to describe this interaction in an editorial recently published in Kidney International (5). In that article, we offered a hypothesis to describe the mechanism by which ischemic injury develops and integrated the findings from our experimental models with those of other investigators as well.

Finally, we have begun to investigate the direct effects of CCB on anoxic and hypoxic injury to freshly isolated renal proximal tubules from the rat since 1) this procedure provides us with a greater experimental mass of tissue than does the cultured tubule preparation and 2) this animal has been used for the majority of in vivo ARF studies. The goal of these experiments is to determine the non-vascular mediated effects of Ca on cell injury and the role of CCB and other maneuvers which prevent or attenuate such injury.

Our first preliminary efforts in the freshly isolated tubule studies showing that verapamil (5 μM) and nifedipine (5 μM) could directly decrease Ca uptake (a major goal of this contract) have been published in abstract form (6). We are currently reevaluating these data in our newer, more viable preparation of tubules. In addition, because acidosis had been shown to ameliorate several kinds of functional and biochemical injury during O_2 deprivation, we investigated this hypothesis in the hypoxic renal tubule preparation.

Acidosis was achieved by adding HCl (0.1 N) to the media just as hypoxia was induced by changing the gas mixture to 100% N_2. In that study (Am J Physiol, in press) (7), acidosis was maintained at pH 6.9 throughout hypoxia and for 60 min of reoxygenation with 95% O_2/5% CO_2. This maneuver reduced morphologic damage, attenuated the rise in LDH release by tubules, reduced ^{45}Ca uptake, and decresaed ^{45}Ca efflux. These was no protective effect on adenine nucleotide levels, however, either during acidosis or reoxygenation. These data are shown in the following figure.



Effect of acidosis on LDH release, morphology (% cell injury), and ^{45}Ca uptake and release during and after hypoxic injury to renal tubules in vitro.

We have also begun preliminary studies using the phospholipase inhibitor, parabromophenacyl bromide (PBPB). Given alone and after hypoxia neither 10 μM nor 100 μM PBPB had any effect on preventing O_2 deprivation injury but when combined with glutathione (GSH, 100 μM), 100 μM PBPB reduced LDH release from over 70 to 60% after anoxia. We are continuing these studies and will compare the results to those achieved with higher doses of PBPB and with mepacrine (100 μM), and chlorpromazine, a putative calmodulin antagonist.

In addition, another goal of our contract was to determine if the xanthine oxidase inhibition, allopurinol, and free radical scavengers would present cellular injury. Using the intact rat, we showed that renal tissue adenine nucleotides were increased to (or toward) normal following a 50 min total renal artery occlusion when 40 mg/kg of allopurinol was administered 8 min prior to ischemia. This dose was not protective to adenine nucleotides if given before 60 min of renal ischemia but in two experiments, 80 mg/kg allopurinol did improve renal cortical adenine nucleotide levels. These preliminary studies principally examined adenine nucleotide levels which are known to fall during ischemic injury. The attributed role of allopurinol is to prevent O_2 free radical formation and it may be through this mechanism that adenine nucleotide pores are preserved. Since adenosine triphosphate (ATP) depletion is a hallmark of O_2 deprivation, its role alone in causing cell injury was also explored.

II. ATP Depletion Alone and Its Effect on Renal Function

Phosphate restriction has been shown to be protective against functional deterioration in animal models of chronic renal failure. Using NMR and other metabolic studies, we demonstrated that remnant kidney is associated with a high metabolic rate and phosphate restriction resulted in the lowering of nephron hypermetabolism in this model. To extend this finding to ARF, we examined the critical role of low phosphate in the pathogenesis of ARF.

Studies were first performed in the isolated rat kidney perfused with a phosphate-free perfusate; no functional deterioration was observed in control as well as during reperfusion after 30 min of ischemia. Since ATP was well preserved in kidneys perfused in the absence of phosphate, experiments were carried out in models of ATP depletion with glycerol or fructose (40 mM) added to the control perfusate. Similarly, renal function was observed during control periods without ischemia or following 30 min of clamp ischemia. Addition of glycerol or fructose resulted in lowering ATP levels to 60% and 30%, respectively. However, physiological parameters of renal function were unaffected. Moreover, during the 70 min of reperfusion after clamp ischemia in this model, inulin clearance was not worsened even though fructose and glycerol treatment prevented recovery of ATP levels to control values. The following are the data from reference 8.

Table II. Renal function following 30 min of ischemia

Experimental group	RPF ml/min/g	C_{In} μl/min/g	FE_{Na} %	V μl/min/g
Control media (n=5)	34.0±0.8	146±43	8.1±1.8	18±4
Media with 40 m*M* glycerol (n=5)	36.8±1.5	125±30	29.6±4.0**	72±14**
Media with 40 m*M* fructose (n=5)	36.8±1.5	163±38	22.1±1.5**	64±15*
Media with 40 m*M* urea (n=5)	34.0±6.4	92±15	25.2±3.1**	34±6*

Results expressed as mean±SEM. *p<0.05; **p<0.01, adjacent vs. control value.

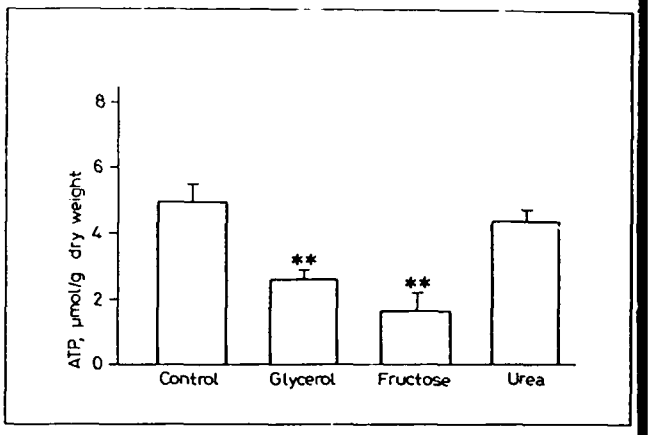


Fig. 3. Tissue levels of ATP after 70 min of reperfusion following 30 min of ischemia. Large bars represent mean of 3–5 determinations for each group, small bars SEM. **p < 0.01, compared with control.

Thus, a 33-60% reduction of renal ATP does not appear to have a deleterious effect on organ function (8). Similar findings were also observed in an in vivo model of ischemic ARF (9). Therefore, the rat kidney can withstand rather low levels of ATP without an appreciable acute effect on organ function. Our data are seemingly at odds with an extensive body of compiled work suggesting a benefit to infusions of ATP-$MgCl_2$ following ischemia. However, there are multiple reasons for this apparent discrepancy. ATP per se may not be important since the half-life of ATP in vivo or during perfusion is very short. ATP is rapidly broken down to other intermediaries that are important. To explore this possibility, fructose-1,6-diphosphate (FDP), a metabolite in the glycolytic pathway, was infused to the isolated kidney perfusion after 40 min of clamping and perfused for 60 min. FDP infusion improved inulin clearance, urine flow and tubular reabsorption of Na. FDP concentration in the perfusate decreased with a proportionate increase in dihydroxyacetone phosphate. Kidney ATP shows no difference during ischemia but is higher after 60 min reperfusion in the treated group. The functional and metabolic findings are substantiated in preliminary in vivo models (10). These findings indicate the importance of substrate provision during reperfusion. More details of these studies are provided in our annual reports and full publications.

Finally, since atrial natriuretic peptide is also vasodilatory and natriuretic, we evaluated its effects in ischemic ARF.

III. Atrial Natriuretic Peptide and ARF

We used the isolated perfused rat kidney to examine the utility of atrial natriuretic factor in the preservation of renal function, to examine the dose-response and to compare the natriuretic response of atriopeptin III (AP-III), a synthetic atrial natriuretic peptide, with furosemide. AP-III was shown to increase GFR and enhance distal delivery of sodium. In addition, the effect of AP-III and furosemide are additive, thus supporting potential therapeutic implications for edematous state and other renal disorders (11). Encouraged by this result, we have extended the study of AP-III to examine its utility in the setting of ischemic ARF. Since the hallmark of ischemic ARF is a profound diminution in GFR and AP-III has been shown to increase GFR independent of glomerular plasma flow, we reasoned that AP-III might provide unique protection against ischemic ARF.

Using ^{31}P NMR, enzymatic assay coupled with isolated rat kidney perfusion

and in vivo functional studies, we have demonstrated the beneficial effect of AP-III in the protection of ischemic ARF in in vitro as well as in vivo models (12). The following two tables (from reference 12) highlight the dose-dependent protective effect of AP-III immediately after ischemia as well as at 48 hr of reperfusion, respectively.

Table I. Effect of AP-III on Renal Function after Ischemia in the Isolated Kidney

	Time	Control	AP-III	AP-III
	min	*0 μg/dl**	*10 μg/dl‡*	*100 μg/dl‡*
Renal plasma flow (*ml/min/g*)	0–30	27.6±3.1	23.8±2.2	25.6±2.7
	30–60	33.5±1.4	36.5±1.4	39.1±1.8§
	60–90	32.2±2.1	34.7±2.0	39.6±2.4§
C_{In} (*μl/min/g*)	0–30	11.6±4.2	3.9±1.6	4.3±0.8
	30–60	17.0±4.7	13.6±2.8	123.4±44.0§
	60–90	24.6±6.2	28.4±8.4	182.6±49.2§
V (*μl/min/g*)	0–30	3.3±0.4	2.6±1.2	1.3±0.4
	30–60	4.8±0.8	6.3±1.6	31.4±8.0§
	60–90	7.1±0.8	10.1±2.1	52.9±12.1‖
T_{Na} (*μmol/min/g*)	0–30	1.3±0.8	0.2±0.1	0.8±0.4
	30–60	2.3±0.8	1.4±0.3	14.8±6.0§
	60–90	2.9±0.9	3.0±1.2	21.2±6.6§
$U_{Na}V$ (*μmol/min/g*)	0–30	0.7±0.3	1.0±0.1	0.5±0.1
	30–60	0.7±0.1	0.9±0.2	3.6±0.8‖
	60–90	0.8±0.2	1.2±0.2	5.6±1.3‖

Results expressed as mean ± SEM. All data normalized per gram of left kidney weight.
* $n = 5$. ‡ $n = 6$. § $P < 0.05$. ‖ $P < 0.01$ vs. control.

Table III. Effect of AP-III Infusion on Recovery of Renal Function over 48 h after 60 min of Ischemia In Vivo

	Time	Control	AP-III
	h	*n = 12*	*n = 12*
C_{Cr} (*μl/min/100 g*)	0–24	60±23	110±18*
	24–48	126±34	219±34*
FR_{Na} (%)	0–24	94.1±1.8	98.3±0.3*
	24–48	93.6±3.0	99.3±0.2*
T_{Na} (*μmol/min/100 g*)	0–24	7±3	15±3*
	24–48	12±3	38±6‡
$U_{Na}V$ (*μmol/min/100 g*)	0–24	0.127±0.013	0.145±0.021
	24–48	0.173±0.024	0.153±0.031
FE_K (%)	0–24	279±49	173±44*
	24–48	115±27	50±11*
U_KV (*μmol/min/100 g*)	0–24	0.42±0.05	0.51±0.06
	24–48	0.40±0.06	0.47±0.03

FE_K, fractional potassium excretion; C_{Cr}, creatinine clearance. Results expressed in mean±SEM.
* $P < 0.05$.
‡ $P < 0.01$ vs. control.

We have also shown that the degree of protection afforded by AP-III was better than that accomplished by verapamil in the same model. Others (13) have also confirmed the beneficial effect of AP-III since our initial presentation at the American Society of Nephrology meetings held in 1986 and the subsequent submission of our paper.

Finally, we have determined that the abnormal renovascular reactivity after ischemia in which paradoxical vasoconstriction is seen during reductions in renal perfusion pressure (RPP) is due to changes in renovascular smooth muscle Ca that could be blocked by CCB. Endothelial cell damage and a concomitant loss of endothelium derived relaxing factor may also contribute to the increased pressor sensitivity of the renal vessels. The following figures are from our recently accepted paper (14) and demonstrate these responses.

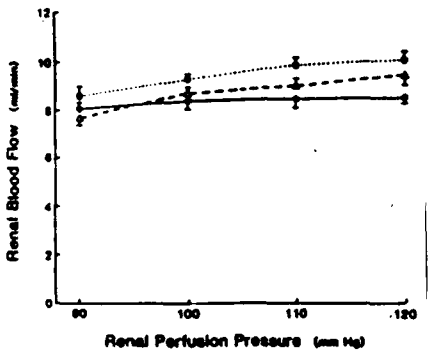


Fig. 1

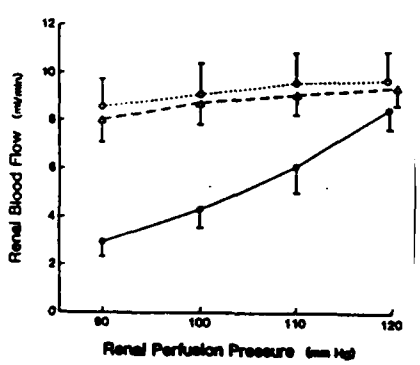


Fig. 2

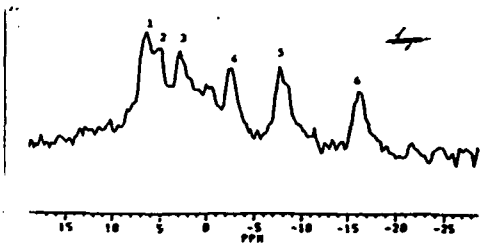
Fig. 1. The response to sequential reduction in RPP prior to (closed circles) and after 90 min intrarenal infusion of verapamil (open triangles) or diltiazem (open circles) in sham-operated rats. Fig. 2. The same data (and symbols) as determined in rats with norepinephrine induced ARF.

IV. Other Studies

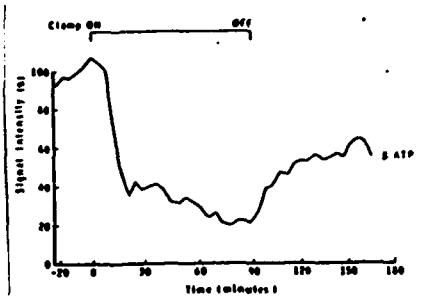
It was of great importance that we characterize the use of the NMR in our laboratory, because this technology was to be used in the contracted studies.

We have applied phosphorus NMR spectroscopy to the in vivo determination of high energy phosphate metabolites (ATP, ADP, AMP), phosphodiesters, intracellular Pi and pH in normal animals and during acute renal failure (ARF). After 2 years of planning, a 1.89 Tesla 30 cm horizontal bore magnet (Bruker/Oxford System Biospec spectrometer) was installed in October of 1985. Preliminary studies were performed in rat kidneys with an NMR probe built in our own Division of Renal Diseases mechanical workshop and electronic laboratory. With this approach, ^{31}P NMR spectrum of a rat kidney in vivo can be obtained in less than 10 min (15-17). These reports are abstracts and although not directly related to this contract, they do help refine the sophistication which we can achieve with the NMR. Furthermore, they are basic investigations designed to discriminate between normal and hypermetabolic states, the latter a characteristic of and a possible contributor to further cellular injury in ARF.

A ^{31}P NMR spectrum of kidney obtained from an anesthetized rat is shown in the following figure. The spectrum was accumulated from 512 scans at 2 second intervals and a pulse width of 12 micro-second (60° pulse). The signals are assigned as follows: 1) unidentified resonance in the sugar phosphates, AMP and the 3-phosphate of 2,3-diphosphoglycerate of the red blood cells; 2) inorganic phosphate and 2-phosphate of 2,3-diphosphoglycerate of red blood cells; 3) an unidentified peak and is thought to be glycerophosphoryl-choline within the inner medulla (plus urine phosphate); 4) gamma-phosphate of ATP with a contribution from beta-phosphate of ADP; 5) alpha-phosphate of ATP, together with a contribution from alpha-phosphate of ADP, and from NAD/NADH; 6) beta-phosphate of ATP. Intrarenal pH was 7.2 (calculated from the chemical shift of Pi).



In a representative experiment, after 40 min of baseline study with the animal kept in the magnet under anesthesia, the renal pedicle was completely occluded for 90 min with a remote clamp. NMR spectra and quantitation were carried out as described earlier. In this experiment only modest regeneration of ATP was observed when the clamp was released after 90 min of ischemia (see following figure).



Parallel experiments were also performed using the isolated perfused rat kidney. The effect of warm ischemia was examined by clamping the perfusion line. Progressively increasing periods of warm ischemia resulted in a decline in renal function when the kidney was subsequently perfused (see following table).

Effect of increasing warm ischemia on renal function and nucleotide content. Function was assessed by perfusion of the isolated kidney for 60 min.

	Warm Ischemia Time (37°C)		
Function During Perfusion	0 min (n=8)	60 min (n=3)	90 min (n=3)
Inulin Clearance (ml/min)	0.60±0.05	0.15±0.04**	0.08±0.05**
% Na^+ Reabsorption	95.90±0.85	53.70±9.40*	25.10±5.40**
Nucleotide Content (μmol/g dry weight)			
ATP	8.18±0.70	4.95±0.80*	1.50±0.50**
ADP	2.18±0.08	2.40±0.25	2.10±0.35
AMP	0.87±0.12	0.80±0.20	1.40±0.60
ATP+ADP+AMP	11.23±1.10	7.15±1.20	5.00±0.70**

* $p<.05$; ** $p<.01$ with respect to the control group. Results are expressed as mean±SEM.

Decline in renal function, as reflected by falling glomerular filtration rate (GFR) (inulin clearance) and fractional Na^+ transport, progressed and renal function reached a very low level with 90 min of ischemia. The results obtained with isolated perfused kidney correspond closely to the time of ischemia required for the development of renal failure in vivo. ATP decreased as expected with the onset of ischemia and total adenine nucleotide content declined to 20 to 40% of control value.

With the above approach of using an isolated perfused rat kidney and in vivo ^{31}P NMR spectroscopy, we then tested the specific protective effect of administration of 1) CCB, or 2) exogenous atrial natriuretic factor, in several ARF studies, as well as the response to 3) lowering intracellular phosphates or ATP, and 4) adding metabolic substrates on this model of ischemic ARF in the rat. As yet, most of this data is in preliminary or abstract form only (10-14-16).

In conclusion, using the above approach with an isolated perfused rat kidney and ^{31}P NMR spectroscopy, we have begun to demonstrate the critical role of energy metabolism in the pathogenesis of ARF and the various possible methods of intervention which can attenuate cell injury in this model.

Summary

The mechanism of renal cellular injury during hypoxia is similar to that seen in combat injury where hypotension, shock and poor tissue perfusion predispose organs to injury.

During hypoxic injury, there is an increase in total tissue Ca; during reperfusion or reoxygenation tissue Ca falls initially and then rises some while later (approximately 1 hr). An increase in ionized Ca has been reported and is likely to be due to redistribution of Ca from internal stores such as those in mitochondria or endoplasmic reticulum.

During hypoxic injury and reperfusion, there can be as much as a 4-fold increase in tissue Ca levels, indicating that Ca must enter the cells and fail to be extruded. LDH release is increased, ATP levels fall and signs of cellular injury can be quantitated morphologically.

CCB treatment prevented Ca influx in isolated tubules. Acidosis (pH 6.9) is also protective but not via any change in ATP levels; however, LDH release is greatly attenuated and morphologic injury is reduced. In ischemic renal injury to normal kidneys in vivo or to the isolated perfused in vitro kidney or in anoxia in cultured tubule studies, CCB delay or prevent functional biochemical and morphologic injury. Calmodulin antagonists also appear to attenuate injury possibly by interfering with Ca-mediated intracellular events. To this end, Ca-mediated phospholipase activation and subsequent membrane permeability changes could participate in cellular injury. The preliminary results of the phospholipase inhibitor studies seem to favor this hypothesis. Even in face of the results from the fructose and glycerol studies, it would still seem advantageous to salvage adenine nucleotides and/or to attenuate further ATP depletion in post-ischemic tissue. Thus, the beneficial effects of fructose 1,6-diphosphate and the adenine nucleotide retention associated with allopurinol treatment suggest that normalization of energy metabolism should be a goal in the treatment of ischemic ARF.

Our anticipation, pointed out in our proposal for continued funding from the U.S. Army Medical Research Acquisition Activity, is that allopurinol may be additively protective to either CCB or acidosis since it raises high energy compounds (18) in a dose-dependent manner. We had proposed that the next investigation examine the effects of multiple drug administration both in vivo and in vitro.

V. Patient Studies

Based on the laboratory and animal studies conducted during this contract, we have proposed that intrarenal atrial natriuretic factor or a verapamil analogue (D-600, gallopamil) could improve GFR in patients already in or at risk for ARF.

If GFR could be increased by just 5 ml/min in each kidney, the need for dialysis would be markedly lessened if not obviated. To this end, we have begun clinical trials in such patients and the approved protocol is attached as Appendix I. The financial assistance from this Army Contract has permitted us to generate the necessary animal data which now makes possible testing the theory in man.

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RESEARCH PROTOCOL

TITLE

A Controlled, Randomized Open Pilot Study to Investigate the Effects of Intra-arterial and Intravenous Atrial Natriuretic Factor (L-364,670) or Gallopamil (D-600) in the Treatment of Acute Renal Failure

SITE

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I. Purpose of the Study

Despite considerable investigative effort and attempts to improve therapy, mortality from acute renal failure (ARF) continues to be high. This is particularly true when ischemia is the primary etiological factor. Mortality in ischemic ARF ranges for 29-85% depending in large part on the nature of the underlying surgical or medical disorder (1-9). In addition, ARF is a major factor in determining the outcome of patients with multiple organ failure. In similarly traumatized patients, those who have developed ARF have had a five-fold higher mortality than those who did not (10).

Attempts to attenuate or reverse ARF and, thereby, reduce mortality have centered around pharmacological, nutritional, and altered dialysis intensity approaches. While the initial reports with each of these therapeutic maneuvers were promising, subsequent studies have indicated that there has been limited improvement in survival (1) with these treatments. As a pharmacological treatment, furosemide has been shown to increase urine flow rate; however, it has not appreciably altered the course or outcome of ARF (11-13). A combination of dopamine and furosemide was reported to produce a diuresis if used early in ARF; unfortunately, the mortality was similar in patients who did and did not have a diuretic response (14). Attention to nutritional factors was focused by the report of Abel, et al. (15). He reported that patients receiving a combination of glucose and amino acids had a 75% survival, whereas those receiving glucose alone had a 44% survival. By contrast, among 30 ARF patients treated parenterally, Feinstein, et al. (16) found a lower nitrogen output in those given glucose plus essential amino acids than those receiving glucose alone or glucose plus essential and nonessential amino acids. Despite the

modest change in nitrogen balance, there was no difference in between survival and catabolism. Several subsequent studies using various combinations of amino acids, glucose and total calorie intake have also shown marginal improvement in nitrogen balance and reduced catabolism but no definitive change in the course of ARF or outcome (17-19). The advent of dialysis initially had a major impact on mortality from ARF. In reports from wartime experience, 90% of casualties with ARF died during WW II compared to an average of 60% in Viet Nam (4, 8, 20). However, attempts to further improve survival using more vigorous dialysis have had mixed results. Several reports which have compared survival using different dialysis techniques in dissimilar time periods have shown lower mortality with more vigorous dialysis in a later time period (21-24). Unfortunately, none of these studies was controlled for time-related variables including newer antibiotics, resuscitative techniques and general improvement in supportive care. Two prospective studies examining dialysis that were structured to maintain predialysis serum creatinine (S Cr) < 5 mg/dl (A) or > 10 mg/dl (B) showed conflicting results (25, 26). The first study, carried out in surgery-trauma patients, showed 36% mortality in A and 80% mortality in B (25). Unfortunately, the follow-up study (26) performed in a larger number of patients with both medical and surgical disorders showed no differences in survival using the A or B dialysis regimens. In summary, considerable clinical experience and numerous studies have not consistently demonstrated a significant impact on ARF survival rates using available pharmacological, nutritional and vigorous dialysis techniques.

The proposed studies, which are based on recent observations using atrial natriuretic factor (ANF) and calcium entry blockers experimentally, are designed to determine if either of these agents can alter the course of clinical ARF and, thereby, reduce morbidity and mortality from this disease. ANF has been demonstrated to reverse ischemic ARF in experimental animals when given after the ischemic insult (27). The mechanism of protection appears to be via a direct increase in glomerular filtration rate (28). Calcium entry blockers also have been shown to attenuate ARF when given following ischemia (29). However, they appear to be protective by diminishing reperfusion injury to the tubular epithelium.

In this study, patients with documented acute renal failure that have not been treated with dialysis will be randomly divided into three groups. Group I will be given intra-arterial or intravenous ANF (L-364,670); Group II will be given a calcium entry blocker (Gallopamil D-600), intra-arterially or intravenously. Group III will receive standard conservative management. Dialysis requirements, morbidity and mortality rates will be compared among the three groups.

II. Background and Rationale

A. Atrial Natriuretic Factor (ANF)

An intriguing and recently described potentially therapeutic agent in acute renal failure is atrial natriuretic factor (ANF). Data on the effect of ANF on ischemic ARF are limited but impressive. Cole and Needleman (30) reported a nearly four-fold improvement in GFR 24 hours after 40-minute renal artery clamp when atriopeptin 24 was infused for 60 minutes after clamp release. Recently, Nakomoto et al (27) examined the effects of ANF given after ischemic insult both in the isolated perfused kidney and in vivo in the rat. ANF, when added at 100 micrograms to the perfusate in vitro for 90 minutes or infused at 0.2 micrograms/kg/minute for 120 minutes in vivo after 60 minutes of renal ischemia improved inulin clearance greater than seven fold in the former and greater than three fold in the latter compared to untreated controls. Moreover, the effect persisted for 48 hours as determined in a separate study where rats treated for 60 minutes with ANF following 60 minutes bilateral renal artery clamping had creatinine clearances more than twice those of untreated controls. The authors suggested that the therapeutic effect of ANF was due to direct increase in the driving force of ultrafiltration, i.e., afferent arteriolar dilatation and efferent arteriolar constriction to increase glomerular ultrafiltration pressure, to the increased tubular fluid flow rate and to an overall decrease in total renovascular resistance. They acknowledge that other undefined factors may also play critical roles.

Conger, et al (31) examined the effects of ANF on glomerular filtration rate after 50 minutes of renal artery clamping in the rat. The authors found that ANF corrects the decrease in glomerular filtration rate (GFR) induced by renal artery (RA) clamping. However, a decline in systemic blood pressure (BP) accompanies the improved GFR. Also, the glomerular mechanism of GFR improvement is unclear. In this study ANF (0.2 micrograms/kg/minute) and D, sufficient to maintain BP >100 mm Hg were infused IV for 240 minutes after 50 minute RA clamp in Munich-Wistar rats. Control (C) rats were infused with saline vehicle. BP, renal blood flow (RBF), GFR , urine flow (V), glomerular plasma flow (QA), single nephron GFR (SNGFR), transcapillary glomerular hydraulic pressure ( ), transcapillary colloid osmotic pressure ( ), and glomerular ultrafiltration coefficient (K) were measured during the final 60 minute infusion. Whole kidney and micropuncture results:

(ANF-D = atrial natriuretic factor with dopamine)

	BP mmHg		RBF ml/minute		GFR ml/minute		V microliters/minute	
C	110	9	4.0	1.5	0.362	0.12	10	10
ANF-D	102	5	7.2	2.6*	1.06	0.34*	87	57*

	QA nl/minute		SNGFR nl/minute		P mmHg		mmHg		K nl/s/mmHg	
C	74	114	32	13	33	4	16	2	.0388	.0215
ANF-D	250	17	57	5*	50	5*	16	1	.0306	.0042

* different at minimum of $p < 0.05$.

The rise in P was the result of a markedly elevated glomerular capillary pressure (P GC) (69 7 in ANF-D vs 49 1 mmHg in C, $p < 0.001$). Conclusions: 1) ANF-D protects BP while improving post-ischemic GFR; 2) the mechanism for increase in GFR is a rise in glomerular capillary pressure.

At the University of Colorado Health Sciences Center (UCHSC), the investigators have infused one patient with intra-renal ANF (L-364,670). The patient was an 81 year old white male status post abdominal aortic aneurysm repair who developed acute renal failure. His baseline serum creatinine was 1.4. On post operative day three his creatinine was 4.5 with a renal failure index of 2.89 and a fractional excretion of sodium of 4.02. His baseline urine output was 26 cc/hour. The patient was catheterized via the left axillary artery. He underwent a four hour infusion of ANF (L-364,670). The initial dose of ANF (L-364,670) was 0.1 microgram/minute. This was slowly increased during the infusion time to 0.5 micrograms/minute for the last hour of the infusion. His urine output increased to a maximum of 100 cc during the first 2 hours post-infusion. The patient remained hemodynamically stable throughout the infusion. He was discharged to home on the 15th hospital day with a serum creatinine of 3.5.

Atrial natriuretic factor is actually a family of small, structurally related peptides (containing 19 to 33 amino acids), isolated from atrial myocytes, which produce vasodilation and natriuresis. Micropuncture studies suggest these peptides cause changes in pre and post glomerular vascular resistance leading to increases of glomerular capillary pressure and single nephron glomerular filtration rate (28). The vasodilatory effect has been shown to be selective for the renal vascular bed at low doses (32,33).

The magnitude of these effects vary with the dose and exact peptide structure. However, several studies have shown an

increase in GFR to be the major consequences of ANF infusion (34). Associated with the elevated GFR are increases in urine flow rate . sodium and postassium excretion, and a decrease in urine osmolality (U osm) without a change in free water clearance (34). The effects of ANF are immediate and sustained (35).

Reports vary as to the effect of ANF on renal blood flow (RBF), but its magnitude is small whether examined in rat or dog (36,37). The observed striking increase in filtration fraction is not completely understood; however, micropuncture data from rats indicate that there is a simultaneous afferent arteriolar dilatation and efferent arteriolar constriction leading to an increase in capillary hydraulic pressure (38). A change in the glomerular ultrafiltration coefficient, however, has not been excluded. The effect of ANF on vascular smooth muscle is of considerable interest. In most vascular beds ANF has a significant depressor effect (34). Most studies have reported a dose-dependent decrease in renovascular resistance (RVR). By contrast, Camargo and associates (39) have shown an increase in RVR in the isolated perfused kidney. However, when the kidney was pre-constricted with angiotensin, norepinephrine, vasopressin or ouabain, ANF was vasorelaxant (40). ANF is more effective in antagonizing the vasoconstriction of angiotensin than norepinephrine (40).

Kim, et al (41) studied the effect of atrial natriuretic factor on rat glomeruli and different renal tubule segments including cortical thick ascending limb and cortical collecting tubule, outer medullary and inner medullary collecting tubules. The effect of ANF was assessed by alteration in adenylate cyclase and cGMP in the various nephron segments in the presence and absence of AVP, PTH, and SCT. ANF significantly increased ($p < 0.001$) cGMP in the glomerulus and inner medullary collecting tubules. Specific ANF binding was observed in the microdissected inner medullary collecting tubule. The authors suggest that ANF may exert a significant physiological role in the glomerulus and inner medullary collecting tubule by increasing cGMP.

There are a number of systemic effects of ANF. Cardiac output, peripheral vascular resistance (PVR) and systemic blood pressure are reduced in animal studies (34). The effect on PVR is more pronounced in hypertensive models (42). ANF has multiple effects on the renin-angiotensin system. ANF lowers renin secretory rate, plasma renin activity, plasma aldosterone via reduction in angiotensin II, as well as the previously mentioned direct vascular antagonism of angiotensin in dogs (43).

Merck, Sharp, and Dohme Research Laboratories studied the effects of ANF (L-364,670 MET ANF and L-364,343) administered intravenously in healthy volunteers and in patients with edema, congestive heart failure, and hypertension. The data are summarized below.

1. Cardiovascular effect of L-364,670 and L-364,343

The effects of both L-364,343 (8-33 Ile-ANF) and L-364,670(8-33 MET ANF) on blood pressure appear to be dose-dependent. Systolic blood pressure is reduced more than diastolic. Infusion of either compound in doses of up to 1.0 microgram/minute for up to 4 hours does not alter blood pressure in volunteers. At doses above one microgram/minute, both compounds reduce blood pressure. A gradual reduction in blood pressure occurred at doses above 10 micrograms/minute in subjects infused with incremental doses (1, 2, 5, 10, 20, and 40 micrograms/minute for 15 minutes each) of L-364,343 and in subjects given a 4-hour constant-dose infusion of 5 micrograms/minute of this compound. Subjects in the L-364,343 studies were given0.5-1.0 micrograms/minute for 2-4 hours or 2.0 micrograms/minute for 2 hours. During the 2.0 microgram/minute infusion, a gradual fall in blood pressure was observed, which was different from placebo at 2 hours (the end of the infusion). The 0.5 microgram/minute and 1.0 microgram/minute doses of L-343,670 did not alter blood pressure.

The effects of L-364,343 and L-364,670 on blood pressure are not clearly related to the serum concentration. The concentration increases to a plateau within minutes of the beginning of the infusion and falls to baseline within minutes after the infusion is stopped. By contrast, studies using incremental dosing, reveal first reduction in blood pressure after the incremental dose to occur 75 minutes into the infusion. During constant dose infusions of 2-5 micrograms/minute, an effect was apparent after 2-3 hours. The hypotensive effect of L-364,343 and L-364,670 persisted for up to one hour after the termination of the infusion. The investigators reported in their manuscript that the magnitude of the reduction in blood pressure after the incremental dose of L-364,343 was proportional to the total dose of the compound (44).

Heart rate was not altered after infusion of L-364,343 or L-364,670 unless blood pressure fell. With either incremental or higher dose infusions, heart rate rose as blood pressure fell and remained elevated while blood pressure remained low. The proposed explanation for the observed increase in heart rate is a reflex tachycardia induced by the fall in blood pressure.

2. Renal effects of L-364,670 and L-364,343

Renal effects of L-364,343 and L-364,670 are apparent at doses that do not alter blood pressure. Doses as low as 0.5 micrograms/minute produce effects on water and electrolyte excretion, effective renal plasma flow, and filtration fraction which are apparent within 1-2 hours. Glomerular filtration rate was not changed at the lower doses and only marginally, if at all, increased at the higher doses. The natriuretic effect of both compounds was not accompanied by kaliuresis. Renal effects of L-364,343 were clearly dose-dependent, with greater

natriuresis and diuresis at the 5.0 microgram/minute dose than at the 0.5 microgram/minute dose. After 2 hours of 5.0 microgram/minute, when the blood pressure had begun to fall, the natriuretic and diuretic effect of L-364,343 was reduced. This is consistent with the preclinical data and suggests that the renal effects of ANF can be attenuated by a fall in blood pressure. It is also possible that some other factor limits the renal effects of atrial polypeptides when they are given for long periods of time. The decrease in urinary excretion of kallikrein and the inhibition os sodium reabsorption in the renal tubules suggests that ANF affects the distal nephron.

In the MSDRL sponsored studies in salt-loaded, renin-suppressed volunteers, there was a tendency for plasma renin activity and aldosterone to be reduced during ANF infusion. The rise in the aldosterone, renin, and angiotensin II levels after the cessation of the infusion suggests that the renin-angiotensin-aldosterone system may have been suppressed during the infusion. Levels of serum and intracellular electrolytes were not altered during the ANF infusions. Renal Na, K-ATPase activity was transiently inhibited. No effect on vasopressin was observed.

3. Effects of L-364,670 in patients with edema

An interim analysis of a study to compare the effects of L-364,670 with furosemide in patients with cirrhosis and ascites and in patients with nephrotic syndrome has been performed. Results are available from six patients, three with cirrhosis and ascites and three with nephrotic syndrome. The results of four hour infusions of L-374,670 starting at a dose of 0.25 micrograms/minute and increasing hourly to 2.5 - 5.0 micrograms/minute were compared with the effects of a single 40 mg injection of furosemide.

At the doses used in this study, L-364,670 reduced systolic blood pressure in all subjects. In the three subjects with nephrotic syndrome, a sudden episode of hypotension and bradycardia (accompanied by syncope in one case) was superimposed on the more gradual reduction in systolic blood pressure. Episodes of hypotension and bradycardia occurred while two patients were receiving 2.5 micrograms/minute and one was receiving 5.0 micrograms/minute.

Preliminary results of this incomplete study show that although L-364,670 has natriuretic and diuretic effects in patients with cirrhosis and ascites and in patients with nephrotic syndrome, it was minimal. The occurrence of abrupt symptomatic hypotension limited the use of the compound in patients with nephrotic syndrome. In addition, the fall in blood pressure probably limited the renal effects of L-364,670. Diuretic and natriuretic effects of a 40 mg dose of furosemide were of far greater magnitude than those of L-364,670.

Limitation of diuretic and natriuretic effect correlated with the lack of substantial increase in GFR and filtration fraction. The

free water clearance which has been seen in normal volunteers occurred in only one patient, and it was not sustained. ANF levels measured at baseline were above normal and increased to high physiological levels with L-364,670 infusions.

4. Effects of L-364,343 and L-364,670 in patients with congestive heart failure

Effects of L-364,343 and L-364,670 on blood pressure and heart rate in patients with congestive heart failure appear to be similar to those seen in normal volunteers. At doses of 0.5 - 1.0 micrograms/minute for up to four hours demonstrate no effects on blood pressure or heart rate. At higher doses, both a gradual reduction in blood pressure and the occasional occurrence of abrupt hypotension have been observed.

L-364,670 and L-364,343 appear to reduce right and left ventricular filling pressures in most patients with congestive heart failure. The dose response relationship or the effect on filling pressures is not clear. Effects on cardiac output have been variable.

Effects of L-364,343 and L-364,670 on renal excretion of water and electrolytes appear to be similar to that seen in volunteers. Both compounds produce a diuretic and natriuretic effect at doses as low as 0.5 micrograms/minute. It is of lesser magnitude than that produced by therapeutic doses of thiazide diuretics. It should be noted that although fractional excretion of sodium doubled during infusion of L-364,670, this would not be expected to produce a clinically useful natriuresis. In patients with congestive heart failure, the effect on kaliuresis is unclear. The dose response relationship of the renal effects of the compounds in patients with congestive heart failure has not been determined.

5. Effects of L-364,670 in patients with hypertension

Studies are underway to assess the effects of L-364,670 in patients with hypertension. The protocol for one study has been altered secondary to the occurrence of three episodes of abrupt hypotension accompanied by bradycardia.

One study compares the effects of L-364,670 in patients on high and low salt diets. Patients in this study stayed in the hospital with carefully controlled sodium intake two days before and during the infusions of L-364,670 and placebo. The data clearly show that the effects of L-364,670 are more prominent in vasoconstricted patients with lower sodium intakes. The dose-response curve for diastolic blood pressure was calculated by the investigators and is shifted to the left when patients take a lower salt diet (MSDRL in-house report).

The dose required to produce natriuresis was lower than the dose that reduced blood pressure. Because of sudden hypotension

accompanied by bradycardia that occurred three times in two patients receiving 0.5 and 1.0 micrograms/minute after 2-3 hours, the study design was altered so that patients received 48 hour infusions of 0.2 micrograms/minute of L-364,670. There was no change in blood pressure but a minimal natriuretic effect was noted, which resulted in a net negative sodium balance of 50-100 mmol after 24 hours.

6. Tolerability and safety

The most common adverse experience was hypotension and/or orthostatic symptoms (17 cases as of 8/86). Six volunteers/patients had orthostatic symptoms with or without documented hypotension. One volunteer had hypotension that developed abruptly and was accompanied by an increase in heart rate. Neither orthostatic symptoms nor hypotension (accompanied by an increase in heart rate) is unexpected following administration of agents such as L-364,343 and L-364,670 which has both vasodilatory and diuretic effects. All of these episodes occurred after subjects received at least two hours of high doses of L-364,670 and L-364,670 (one 1.5 micrograms/minute, one 3.0 micrograms/minute, four 5.0 micrograms/minute and one 40 micrograms/minute). The occurrence of these symptoms in 4 out of 7 cases after the infusion was terminated is not surprising. Persistence of the hypotensive effect of L-364,343 and L-364,670 after the termination of infusion which was noted in these patients.

An unexpected finding in the clinical studies of L-364,343 and L-364,670 was the occurrence of abrupt hypotension accompanied by bradycardia. Eleven such events have occurred in 10 subjects during 125 infusions of L-364,343 and L-364,670 in 81 subjects. These events have been experienced by volunteers and by patients with edema, congestive heart failure and hypertension.

Abrupt hypotension occurred during or after (3 cases) infusions of L-364,343 and L-364,670. In volunteers and patients with edematous states or congestive heart failure, abrupt hypotension occurred only during infusion doses of 2 micrograms/minute or higher. The infusion duration ranged from 70 to 238 minutes with some tendency for the higher doses to produce hypotension earlier. In hypertensive patients, especially those on a restricted sodium diet, the dose which produces abrupt hypotension appears to be lower - i.e. 0.5 micrograms/minute to 1.0 micrograms/minute. Heart rate during the episodes ranged from 48 beats/minute to 54 beats/minute. This was reduced from baseline and blood pressure measurements immediately prior to the event. With one exception, where electrocardiographic monitoring was done, the patients were in sinus rhythm during the episode. One hypertensive patient experienced a 5 second period of asystole 90 minutes after the L-364,670 infusion was completed. All patients were symptomatic during the episodes, one patient fainted. All patients quickly responded to lying down, inversion or fluid supplementation and recovered fully.

Several other adverse experiences have been reported in subjects receiving L-364,343 and L-364,670. Of these, headache (two subjects), palpitations, and sweating may have been manifestations of undocumented hypotension. None of these were considered serious. One patient developed fever 48 hours after the infusion and had elevated liver enzymes one week after the infusion. Neither of these adverse experiences was considered to be drug related.

Upon recommendation from MSRDL, the synthetically manufactured human peptide ANF (L-364,670) will be used in this study.

B. Calcium entry blockers

It has been demonstrated that intra-cellular calcuim (Ca) has a critical role in cell survival following ischemic injury. Both in vivo and in vitro experiments have demonstrated that alterations in calcium homeostasis can convert a reversible form of cell injury to an irreversible lesion (45). Immediately following short-term anoxia, cytosolic free calcium is shown to increase from 40 to 60 nM. In other experiments in which isolated proximal tubules were subjected to varying degrees of anoxia, non-lethally injured tubules showed an increase in cell calcium that is partially reversible following reoxygenation (46, 47). These data strongly suggest that changes in calcium metabolism precede cell death.

Two mechanisms - a redistribution of intracellular Ca and disturbances in transcellular Ca flux - appear to play important roles in setting cytosolic Ca levels following ischemia. Evidence for redistribution comes from studies of isolated perfused cardiac septum subjected to high flow hypoxia (48). Resting tension was not affected by hypoxia initially. However, within five minutes resting tension increased without a concomitant rise in total cell Ca. Since resting tension is function of sarcoplasmic Ca content and resting tension increased without a simultaneous rise in total cell Ca, the implication is that there was a shift of Ca from cytosol to the sarcoplasm. Evidence for a Ca efflux and influx changes to alter total cell and cytosolic free Ca comes in part from a recent study demonstrating that Ca uptake by a suspension of proximal tubules is increased following 30 min of anoxia (49). This increase is the result of an increase in the size of both exchangeable cellular Ca pools (glycocalyx and intracellular). Other studies in LLC-MK2 cells have demonstrated that as cytosolic free Ca increases and ATP decreases during anoxia, Ca efflux increased 2.5 times (50). Thus, it does not seem that the increased cytosolic free Ca results from a decreased ability of the cells to actively extrude Ca.

Recent studies in cultures of individual nephron segments have been able to demonstrate deleterious effects of extracellular Ca on the recovery of cells from an anoxic insult (51). Following anoxia, cells incubated in Ca-free media for 2 hours, thereafter showed a 46% viability at 48 hours compared to 100% cell death in an incubated Ca-containing medium.

The mechanistic role of cell Ca in ischemic cell injury therefore appears to be related to the consequences of increased intracellular Ca. Ca is present in the extracellular fluid at concentrations on the order of 10-3 M, while cytosolic content is in the range of 10T-7 M (52). An increase in the normally low permeability of the plasma membrane or failure of transport mechanisms to remove cytosolic Ca leads to cellular Ca overload. Intracellular Ca overload causes detrimental effects, since several cellular processes depend on cytosolic levels of cell Ca. The deleterious effects of Ca overload on mitochondrial function have been well established (53-57). By competing with oxidative phosphorylation, mitochondrial Ca uptake in effect uncouples this process and limits ATP production (57). Ca produces permeability alterations in the inner mitochondrial membrane by activating membrane phospholipase; Ca has also been shown to precipitate in mitochondrial matrix (57).

Several current investigations are being performed to examine changes in cell membrane permeability, endoplasmic reticulum function and cellular transport processes as a consequence of increases in cytosolic Ca.

Verapamil has been shown to prevent cellular Ca overload in suspensions of proximal tubules and increase cell survival of nephron segment cultures following anoxia (58). Both verapamil and nifedipine normalize the glycocalyx Ca uptake rate and exchangeable Ca pool following anoxia. Verapamil also attenuates the morphological damage associated with anoxia (59). A number of <u>in vivo</u> studies also provide evidence that CEB are protective in ischemic ARF. When verapamil was infused prior to 70-min renal clamp ischemia in rats. GFR was nearly three-fold higher than in control animals 24-hour post-clamp release (60). Similar results were reported by Papadimitriou et al (61) when verapamil pre-treatment was given to dogs undergoing 60-min renal artery clamp. While Malis et al (62) attributed the protective effect of verapamil pre-infusion in norepinephrine ARF to a vasodilator rather than epithelial cell response, other vasodilators have not been found to be protective (63-65). Thus, an effect of verapamil to attenuate cellular ischemic injury cannot be excluded. No protection was afforded by verapamil when given prior to or following 40-min renal artery clamp according to those same investigators (62). However, in a norepinephrine-ARF dog model, Burke et al (66) found not only pre-infusion but post-norepinephrine infusion of verapamil or nifedipine was capable of improving GFR by several-fold at 1 and 24 hours after ARF induction. This was the first <u>in vivo</u> demonstration that pharmacological intervention following ischemic insult improved the course of renal failure.

Human studies with CEB are limited. Wagner et al (67) have reported that diltiazem when given either in the kidney perfusate and recipient or in the recipient alone results in significantly improved early graft function. On the other hand, use of verapamil IV. in unstable patients with azotemia and heart failure did not improve renal function (68). However, it is difficult to separate significant pre-renal azotemia from ischemic ARF in these patients.

1. Pharmacology of Gallopamil (D-600)

The calcium entry blocker Gallopamil (D-600), introduced in 1983, and widely used in Europe, is a methoxy derivative of verapamil which has pharmacokinetic and pharmacodynamic properties that appear to be identical those of verapamil (69). Knoll laboratory, manufacturer of Gallopamil (D-600),has agreed to provide funding for this study. Experimental and clinical studies have shown that at similar doses Gallopamil (D-600) is three to five times more potent than verapamil. Gallopamil (D-600) is available for administration in the oral and intravenous form. When administered intravenously Gallopamil (D-600) is 90% bound to plasma proteins. Its excretion half-life is 3.5 hours; however, its bioeffective half-life is approximately 30 minutes due to its rapid metabolism by the liver (69). Approximately 60% of an administered dose appears in the urine when given intravenously. The proportion of unchanged drug in the urine is between 0.4% and 2% of the dose administered. The primary metabolite is a secondary amine produced by N-dealkylation.

2. Cardiovascular effects of Gallopamil (D-600)

The effects of Gallopamil (D-600) that have been measured are predominantly in the cardiovascular system. Stimulus-response coupling of vascular and cardiac muscle is attenuated (70). Cardiac output after intravenous injection usually remains unchanged even in patients with coronary artery disease and poor left ventricular function since the negative inotropic effect is largely compensated for by the reduction in afterload (71). Gallopamil (D-600) reduces cardiac electrical automaticity (72) and appears to have a lesser tendency to produce slowing of the heart rate than other similar calcium antagonists (73). Like other group A calcium entry blockers, Gallopamil (D-600) is a direct vascular smooth muscle relaxant capable of reducing systemic arterial pressure. Following intravenous injection of 2 mg of Gallopamil (D-600) systolic blood pressure was reduced by a mean 17 mmHg while diastolic blood pressure fell by a mean of 4 mmHg in stable patients with coronary artery disease and normal volunteers (74).

3. Renal effects of Gallopamil (D-600)

Gallopamil (D-600) has been studied in ischemic tubular cell injury. Balogh and Kovach demonstrated that pretreatment with Gallopamil (D-600) protects the kidney from ischemic injury of hemorrhagic shock (75). Since its known effects on transcellular calcium flux are identical to verapamil, it is assumed that other protective roles in ischemic cell injury will mimic that demonstrated for verapamil and other Group A calcium antagonists.

4. Tolerability and safety

The major side effects and safety considerations of Gallopamil (D-600) are those of all Group A calcium antagonists which are

hypotension, bradycardia, asystole, and congestive heart failure. Hypotension occurs as a function of dose and has been reported in 2.9% of patients receiving standard intravenous doses. Gallopamil (D-600) affects the SA and AV nodes. Heart block and asystole are rare and more likely to occur in elderly patients with sick sinus syndrome. Bradycardia, i.e., ventricular rate <60/minute, has been reported in 1.2% of patients receiving Group A calcium antagonists. Heart failure has been reported only in patients with pre-existing left ventricular dysfunction.

In patients with Duchenne's muscular dystrophy, calcium entry blockers may precipitate respiratory failure. Gallopamil (D-600) also may produce increased intracranial pressure in patients with intracranial neoplasms, hemorrhage or trauma.

IV. Work Proposed

A. Objectives

1. To investigate the safety and efficacy of intra-arterial infusion of ANF (L-364,670) or Gallopamil (D-600) on renal function in patients with acute renal failure.

2. To investigate the safety and efficacy of intravenous infusion of ANF (L-354,670) or Gallopamil (D-600) on renal function in patients with acute renal failure.

3. To investigate ANF (L-364,670) or Gallopamil (D-600) levels in patients with acute renal failure who are treated with these agents.

B. Patient Definition

1. Inclusion criteria

a) The presence of an appropriate clinical setting suggesting ischemic or nephrotoxic ARF

b) Serum creatinine > 2 mg/dl or GFR < 70 ml/minute

c) Urine sodium or urine chloride > 20 mmol/L in the absence of diuretics or urinary tract obstruction

d) Renal failure index > 1

e) Fractional excretion of sodium > 1%

f) U/P creatinine > 40

g) U/P osm < 2

h) Renal failure casts in urinary sediment

If there is difficulty establishing volume status in a patient, a rapid intravenous infusion of 300 ml of 0.9% saline will be used. Ultrasound examination may be used to exclude obstruction in cases where the determination cannot be made clinically.

2. Exclusion criteria:

a) History of hypersensitivity to peptide-like drugs, local anesthetics, heparin or radiocontrast material.

b) Age less than 18.

c) Pregnant women and women who have had unprotected intercourse within the previous 30 days unless a negative UCG is obtained.

d) Clinically significant urinary tract obstruction or volume depletion.

e) Myocardial infarction within the previous five days.

f) Baseline pulse < 60 beats/minute or clinically significant arrhythmias.

g) Unable to maintain systolic blood pressure > 90 mm Hg on dopamine at 10 micrograms/kg/minute.

h) Significant coagulopathy as manifested by platelet count less than 50,000 or prothrombin time/partial thromboplastin values more than 25% above control values.

i) Contraindication to femoral or brachial arterial catheterization.

j) Other investigational agents in the three weeks preceding enrollment.

k) Active septicemia unless treated for 72 hours.

l) Persistent congestive heart failure.

m) Patients who have been dialyzed.

n) Patients with any other condition or therapy which in the opinion of the investigator might pose a risk to the patient.

C. Study Design

This is a randomized, controlled open pilot study of 60 patients with acute renal failure. The protocol is designed to include three groups of patients (twenty patients per group). When acute renal failure has been established using the aforementioned criteria, the patient will be randomized to Group I, II, or

III. Group I will receive ANF (L-364,670) intra-arterially or intravenously. Group II will receive Gallopamil (D-600) intra-arterially or intravenously. Patients will be assigned to the intrarenal or the intravenous route of administration using pre-determined criteria given below. Group III, consisting of conservatively treated patients will serve as a control group for the natural history of the disease. The study will actually proceed in two phases: the dose determining phase and the study phase.

1. Dose determining phase

Before initiation of the study phase, a dose determining phase will be carried out. Patients who meet all the inclusion and exclusion criteria will have: 1) a complete history and physical examination; 2) baseline laboratory data: CBC with differential and platelet count, PT/PTT, SMA-20, BUN, creatinine, urinalysis with urine electrolytes, urea and creatinine, calculation of the renal failure index and the fractional excretion of sodium, EKG; 3) creatinine clearances at 2 and 24 hours post-infusion; 4) serum BUN's and creatinines at time 12 and 24 hours.

Patients who will be treated with intrarenal drug infusions will be transported to the radiology department where the intrarenal catheters will be placed. Patients will have bilateral arterial catheters advanced under fluoroscopic guidance to the renal arteries. Position will be confirmed by an injection of 15 ml or less of contrast media. The patient will then be taken to the intensive care unit for the infusion of ANF (L-364,670) or Gallopamil (D-600). Simultaneously, each patient will also receive 0.8 mg/kg/24 hours of furosemide intravenously.

Three to five patients each will be given intrarenal ANF (L-364,670) or Gallopamil (D-600) at progressing doses detailed below. The dosage will be adjusted based on the clinical response of the patient. Clinical response is defined as:

1) increase in urine output by at least 5 ml per hour and an hourly urine output of at least 20 ml per hour

2) increase in creatinine clearance of at least 25% and a creatinine clearance > 15 ml/minute at either 4 hours or 24 hours

3) maintenance of systolic blood pressure > 90 mmHg, and heart rate > 50 beats/minute (intravenous dopamine will be administered up to 40 micrograms/kg/minute as necessary)

At the end of the infusion the renal artery catheters will be removed and hemostasis will be observed. The patient will remain in the intensive care unit for the next 24 hours for monitoring and observation. Vital signs and urine outputs will be recorded hourly. The intravenous furosemide will continue for 24 hours.

Based on the preliminary studies performed at the Merck, Sharp, and Dohme research laboratories, the initial intra-arterial dosage of ANF (L-364,670) will be 0.1 micrograms/minute. The allowable dose range of ANF (L-364,670) will be 0.05 micrograms/minute to 0.5 micrograms/minute. A maximum dose of 1 microgram/minute and duration of four hours can be used.

Knoll Laboratories recommends that a dose of 40 micrograms/minute be used as the baseline intra-renal dose of Gallopamil (D-600). The maximum Gallopamil (D-600) dose which may be used will be 50 micrograms/minute and a duration of four hours may be used.

Assignment to the intravenous route of administration will not be random. The investigators acknowledge that not all patients who meet the inclusion and exclusion criteria can safely undergo renal artery catheterization. Therefore, the following criteria have been established to assign the patient to the intravenous route of administration:

1) Platelet count < 50,000 or prothrombin/partial thromboplastin > 25% above controls

2) Anatomic contraindications to catheter placement in the renal arteries such as congenital abnormalities, atherosclerotic disease, or reconstructive impediments.

As in the patients treated with the intrarenal approach, patients treated intravenously will have: 1) a complete history and physical examination; 2) baseline laboratory data: CBC with differential and platelet count, PT/PTT, SMA-20, BUN, creatinine, urinalysis with urine electrolytes, urea and creatinine, calculation of the renal failure index and the fractional excretion of sodium, EKG; 3) creatinine clearances at 2 and 24 hours post-infusion; 4) serum BUN's and creatinines at time 12 and 24 hours.

The patient will be in the intensive care unit for the intravenous infusion of ANF (L-364,670) or Gallopamil (D-600). All patients will receive 0.8 mg/kg/24 hours of intravenous furosemide and intravenous dopamine at the initiation of the infusion. If systolic blood pressure cannot be maintained with a dopamine drip up to 40 micrograms/kg/minute, the study will be discontinued.

Three to five patients each will be given intravenous ANF (L-364,670) or Gallopamil (D-600) at progressing doses outlined below. As mentioned above, the study doses will be adjusted based on the clinical response of the patient.

The intravenous infusion of ANF (L-364,670) or Gallopamil (D-600) will be over a 12-24 hour period. The furosemide will continue for 24 hours. Since there have been reports of latent hypotension occurring after the infusion of ANF (L-364,670),

these patients will continue to receive intravenous dopamine to maintain systolic blood pressure > 90 mmHg when indicated. All patients will remain in the intensive care unit for the next 24 hours for monitoring and observation. Vital signs and urine outputs will be recorded hourly.

Merck, Sharp, and Dohme research laboratories (MSDRL) preliminary data indicate that the 0.5-1.0 microgram/minute dose of ANF (L-364,670) does not produce hypotension and therefore will be the initial dose used in the dose determining phase. MSDRL data also suggest that a gradual reduction in systolic blood pressure occurs at doses of 2.0 micrograms/minute. The maximum dose to be given intravenously will be 5.0 micrograms/minute with dopamine systemic blood pressure support as necessary up to 40 micrograms/kg/minute.

Knoll laboratories reports that Gallopamil (D-600) is three to five times more potent than verapamil and recommends an intravenous infusion of Gallopamil (D-600) of 100 micrograms/minute. The maximum dose to be given will be 160 micrograms/minute.

The dose which satisfies the previously outlined clinical response criteria by increasing GFR and urine output without producing negative systemic hemodynamic effects will be chosen as the dose for the study phase.

2. Study phase

After completion of the dose determining phase, the randomized, controlled study phase will begin. Twenty patients will be randomized to Group I, II, and III. Those randomized to Group I will receive ANF (L-364,670) intra-arterially or intravenously; Group II will receive Gallopamil (D-600) intra-arterially or intravenously. Group III will consist of conservatively treated patients and will serve as the control group.

All patients satisfying the inclusion and exclusion criteria will undergo identical baseline, pre- and post-study procedures as outlined in the dose determining phase. Specifically, they will have: 1) a complete history and physical examination; 2) baseline laboratory data: CBC with differential and platelet count, PT/PTT, SMA-20, BUN, creatinine, urinalysis with urine electrolytes, urea and creatinine, calculation of the renal failure index and the fractional excretion of sodium, EKG; 3) creatinine clearances at 2 and 24 hours post-infusion; 4) serum BUN's and creatinines at time 12 and 24 hours.

Patients who will be treated with intrarenal drug infusions will have bilateral renal arterial catheters placed and position confirmed as described in the dose determining phase. The infusion of ANF (L-364,670) or Gallopamil (D-600) will occur in the intensive care unit. Simultaneously, each patient will also receive 0.8 mg/kg/24 hours of furosemide intravenously.

The intrarenal and intravenous doses of ANF (L-364,670) and Gallopamil (D-600) to be used during the study phase will be based on data obtained during the dose determining phase and data from MSDRL and Knoll laboratories. These doses are not exact and may be manipulated according to the clinical response of the patient as in the dose determining phase. To reiterate, clinical response is defined as:

1) increase in urine output by at least 5 ml/hour and an increase in urine output of at least 20 ml/hour

2) increase in creatinine clearance of at least 25% and a creatinine clearance > 15 ml/minute at either 4 hours or 24 hours post-infusion

3) maintenance of systolic blood pressure > 90 mmHg, and heart rate > 50 beats/minute (intravenous dopamine will be administered up to 40 micrograms/minute as necessary)

The initial intrarenal dose of ANF (L-364,670) will be 0.05 micrograms/minute - 0.5 micrograms/minute. This may be increased to a maximum dose of 1.0 microgram/minute with a duration of 4 hours.

The initial intrarenal dose of Gallopamil (D-600) will be 40 micrograms/minute and may be increased to 50 micrograms/minute with a maximum duration of 4 hours.

At the end of the infusion the renal artery catheters will be removed and hemostasis will be observed. The patient will remain in the intensive care unit for the next 24 hours for monitoring and observation. The intravenous furosemide will continue for 24 hours. Vital signs and urine outputs will be recorded hourly.

As stated in the dose determining phase, assignment to the intravenous route of administration will not be random. The criteria established to assign the patient to the intravenous route of administration are:

1) Platelet count < 50,000 or prothrombin/partial thromboplastin > 25% above controls

2) Anatomic contraindications to catheter placement in the renal arteries such as congenital abnormalities, atherosclerotic disease, or reconstructive impediments.

Patients treated with the intravenous approach will have the baseline, pre- and post-study procedures as stated above. They will be in the intensive care unit for the intravenous infusion of ANF (L-364,670) of Gallopamil (D-600). All patients will receive 0.8 mg/kg/24 hours of intravenous furosemide and intravenous dopamine at the initiation of the infusion. If systolic blood pressure cannot be maintained with a dopamine drip up to 40 micrograms/kg/minute, the study will be discontinued.

The intravenous infusion of ANF (L-364,670) or Gallopamil (D-600) will be over a 12-24 hour period. The furosemide will continue for 24 hours. The intravenous dopamine will continue as necessary to support systolic blood pressure > 90 mmHg. The patient will remain in the intensive care unit for the next 24 hours for monitoring and observation. Vital signs and urine outputs will be recorded hourly.

The intravenous infusion of ANF (L-364,670) will begin at 0.5-1.0 micrograms/minute. The maximum dose given intravenously will be 5.0 microgram/minute.

The initial Gallopamil (D-600) intravenous dose will be 100 micrograms/minute. The maximum dose to be given is 160 micrograms/minute.

As with the intrarenal infusions, the doses of ANF (L-364,670) or Gallopamil (D-600) may be adjusted according to the clinical response and without producing negative systemic hemodynamic effects in the patient.

Patients in the control group will receive standard medical care. They will undergo the baseline, pre- and post-study procedures but will not undergo arterial or intravenous catheterization. They will receive 0.8 mg/kg/24 hours of furosemide.

4. Data analysis

Analysis of the data derived from this study will be performed by by computer techniques available in Dr. Conger's laboratory. The primary objective of this study as well as safety is to investigate the efficacy of intra-renal arterial infusion of ANF or Gallopamil (D-600) in patients with ischemic acute renal failure by examining changes in GFR as measured by creatinine clearance.

Based on the entrance criteria, most patients entering the study are expected to have GFR's below 5 ml/minute. A positive clinical response is defined in this study as an increase in creatinine clearance of > 50 % and a creatinine clearance of at least 15 ml/minute. At this level of renal function most patients will no longer be at risk for dialysis.

Assuming an observed response rate in the control group between 0 and 40%, 10 patients per group will be sufficient to detect a difference between the control and ANF or Gallopamil (D-600) groups in response rate of approximately 60 percentage points, with 80% power.

As a difference in the response rate of this magnitude is not expected, this study is considered a pilot study only and no definitive conclusions will be drawn.

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